GLUCOCEREBROSIDE TREATMENT OF DISEASE

REFERENCE TO RELATED PATENT APPLICATIONS

[0001] This application is a continuation-in-part of U.S. Patent Application No. 10/375,906, filed on February 27, 2003, entitled "Regulation of Immune Responses by Manipulation of Intermediary Metabolite Levels." The content of the aforementioned patent application is hereby incorporated by reference, in its entirety.

FIELD OF THE INVENTION

[0002] This invention relates to the use of a naturally occurring, mammalian intermediary metabolite or T cell receptor ligand, preferably Glucocerebroside, for the treatment of immune mediated or immune related diseases or disorders, infectious diseases, metabolic disorders and cancer in mammalian subjects.

[0003] All patents, patent applications, patent publications, scientific articles, and the like, are hereby incorporated by reference in their entirety in order to describe more fully the state of the art to which the present invention pertains.

BACKGROUND OF THE INVENTION

[0004] Various methods have been described for the treatment of immune-related or immune mediated disorders or diseases, infectious diseases, metabolic disorders and different types of cancer in mammalian subjects. One of these methods involves the modulation of immune responses in a subject. This includes the down regulation of the immune response system using procedures or combinations of procedures for

producing and applying a new and unexpected immune modulation termed selective immune down regulation (SIDR). Immunological modulation is an artificially induced variation in a subject's immune system in response to the introduction of reagents, procedures and processes. These procedures have been described in detail in U.S. Patent Application No. 08/808,629, filed on February 28, 1997, U.S. Patent Application No. 10/377,628, filed on March 4, 2003, U.S. Application No. 10/377,603, filed on March 4, 2003, U.S. Patent Application No. 09/447,704, filed on February 28, 1997, U.S. Application No. 10/385,440, filed on May 9, 2001, and U.S. Application No. 09/356,294, filed on July 16, 1999. Each if the foregoing patents are incorporated by reference in their entirety in the present application and may further be used in conjunction with the present invention.

[0005] Other methods describe the use of educated or treated cells in the treatment of a variety of diseases. Specifically, the methods are directed to the manipulation of the NKT cell population in a subject that results in the modulation of the Th1/Th2 balance toward anti-inflammatory or pro-inflammatory cytokine producing cells. A detailed description of these inventions have been disclosed in U.S. Patent Application entitled "Educated NKT Cells and Their Uses in the Treatment of Immune-Related Disorders" by Yaron Ilan et al., filed on June 25, 2003 (Application No. not yet assigned), PCT Application No. IL01/01197, filed on December 24, 2001, and U.S. Application No. 10/375,906, filed on February 27, 2003. Each of the foregoing patents is incorporated by reference in its entirety in the present application and may further be used in conjunction with the present invention.

[0006] The present invention provides a new method for the treatment of immune-related or immune mediated disorders or diseases, infectious diseases, metabolic disorders and different types of cancer in mammalian subjects, and preferably, human subjects. This method involves the

administration of an intermediary metabolite or a T cell receptor ligand to a subject. Other methods disclosed herein use this administration step along with other procedures described in prior patent applications incorporated by reference herein. These methods are further described in detail below.

[0007] An intermediary metabolite or a T cell receptor ligand is used in the present invention for the treatment of disease. The intermediary metabolite or the T cell receptor ligand may comprise a lipid or conjugated biomolecule. The conjugated biomolecule may in turn comprise a glycolipid, lipoprotein, apolipoprotein, or glycoprotein other than antibodies, cytokines, or hormones. A glycolipid may comprise a monosaccharide ceramide. A monosaccharide ceramide may comprise a glucosylceramide or galactosylceramide.

[0008] Glucosylceramide is a naturally occurring glycolipid consisting of ceramide, to which glucose is attached. A ceramide, which is a sphingosine and a fatty acid, is the structural unit common to all sphingolipids. Sphingolipids have a variety of cellular functions. These include membrane structural roles and cell signaling participation. (Sullard et al., 2000 Journal of Mass Spectrometry 35: 347-353.) Glucosylceramide is made by the enzyme glucosylceramide synthase which attaches the two molecules together. (see Figure 1 and Figure 2). An example of a glucosylceramide includes glucocerebroside, or a glucocerebroside analogue or derivative.

[0009] The genetic disease Gaucher's Disease is characterized by an accumulation of glucosylceramide. In the treatment of this disorder by appropriate enzyme therapy, the excess glucosylceramide is degraded. Two side effects of this treatment have been noted. In the course of this treatment, chronic active hepatitis associated with Hepatitis C virus infection was exacerbated. Additionally, certain patients (with pre-diabetic conditions) experienced the development of diabetic conditions, indicating an onset of Type II Diabetes. These observations further directly confirm that in human

subjects, Glucosylceramide levels regulate the onset of immune-mediated or immune- regulated disorders or diseases.

SUMMARY OF THE INVENTION

[0010] This invention relates to the use of a naturally occurring, mammalian intermediary metabolite or T cell receptor ligand, for the treatment of immune mediated or immune related diseases or disorders, infectious diseases, metabolic disorders and cancer in mammalian subjects. In a preferred embodiment, such mammalian subjects are human beings.

[0011] This invention provides a process for treating a disease in a mammalian subject comprising administering to the subject an effective amount of a mammalian intermediary metabolite.

[0012] This invention further provides a process for treating a disease in a mammalian subject comprising administering to said subject an effective amount of a T cell receptor ligand.

[0013] The present invention also provides a process for treating a disease in a mammalian subject comprising administering to said subject an effective amount of Glucocerebroside.

[0014] Another aspect of the present invention provides for the treatment of a disease in a mammalian subject comprising the ex vivo treating or educating of cells obtained from the mammalian subject. The cells are treated or educated with an effective amount of the intermediary metabolite.

The treated or educated cells are then re-administered to the subject.

[0015] Another aspect of the present invention provides for the treatment of a disease in a mammalian subject comprising the ex vivo treating or educating of cells obtained from the mammalian subject. The cells are treated or educated with an effective amount of the T cell receptor ligand. The treated or educated cells are then re-administered to the subject.

[0016] Yet another aspect of the present invention provides for the treatment of a disease in a mammalian subject comprising the ex vivo

treating or educating of cells obtained from the mammalian subject. The cells are treated or educated with an effective amount of Glucocerebroside. The treated or educated cells are then re-administered to the subject.

[0017] The present invention also relates to the treatment of a disease in a mammalian subject comprising the re-administration of treated or educated cells to the subject, and the direct administration to said subject of an effective amount of intermediary metabolite.

[0018] The present invention provides for the treatment of a disease in a mammalian subject comprising the re-administration of treated or educated cells to the subject, and the direct administration to said subject of an effective amount of T cell receptor ligand.

[0019] The present invention also relates to the treatment of a disease in a mammalian subject comprising the re-administration of treated or educated cells to the subject, and the direct administration to said subject of an effective amount of Glucocerebroside.

[0020] Numerous other aspects and embodiments of the present invention are described in further detail below.

BRIEF DESCRIPTION OF THE FIGURES

[0021] **Figure 1** shows the chemical structure of Glucocerebroside.

[0022] **Figure 2** shows the pathway to Glucosylceramide synthesis.

[0023] **Figure 3** shows the effect of Glucocerebroside on liver enzymes.

[0024] **Figure 4** shows liver histological sections prepared from mice.

[0025] **Figure 5** shows the effect of Glucocerebroside on Serum IFNγ.

[0026] **Figure 6** shows the effect of Glucocerebroside on Serum IL2.

[0027] **Figure 7** shows the effect of Glucocerebroside on Serum IL12.

[0028] **Figure 8** shows the effect of Glucocerebroside on Serum IL-4.

[0029] **Figure 9** shows the effect of Glucocerebroside on Serum IL10.

[0030] **Figure 10** shows the effect of Glucocerebroside on liver NKT cells.

[0031] **Figure 11** shows the effect of Glucocerebroside on spleen NKT cells.

[0032] **Figure 12** shows the effect of Glucocerebroside on NKT cell proliferation in vitro.

[0033] **Figure 13** shows colonic histological sections prepared from mice.

[0034] **Figure 14** shows the effect of Glucocerebroside on Macroscopic Colitis Score.

[0035] **Figure 15** shows the effect of Glucocerebroside on Microscopic Colitis Score

[0036] **Figure 16** shows the effect of Glucocerebroside on spleen CD4/CD8 ratio.

[0037] **Figure 17** shows the effect of Glucocerebroside on liver CD4/CD8 ratio.

[0038] **Figure 18** shows the effect of Glucocerebroside on serum cytokine levels.

[0039] **Figure 19** shows a Glucose Tolerance Test for Glucocerebroside treatment.

[0040] **Figure 20** shows a Glucose Tolerance Test for Glucocerebroside treatment.

[0041] **Figure 21** shows the effect of Glucocerebroside on tumor size.

DETAILED DESCRIPTION OF THE INVENTION

[0042] The present invention provides methods for the treatment of a disease in a mammalian subject by the administration of an effective amount of an intermediary metabolite to the subject. The intermediary metabolite includes, but is not limited to a Tcell receptor ligand, a lipid, a polar lipid, a conjugated biomolecule, a glycolipid, a lipoprotein, an apolipoprotein, a glycoprotein, a monosaccharide or polysaccharide ceramide, a glucosylceramide, a galactosylceramide, a glucocerebroside, a glucocerebroside analogue or derivative, a sphingosine, a sphingolipid or a ceramide. In a preferred embodiment of the invention, the mammalian subject is a human being.

[0043] The present invention describes a method for treating a disease where regulatory, immune-regulatory or NKT cells are obtained from the subject to be treated, or from another subject, and are educated or treated ex vivo. The cells are treated or educated by the presence of intermediary metabolite, antigens or epitopes, and antigen presenting cells, or any combination thereof. The treated or educated cells are then re-administered to the subject. The cells may be administered to the subject by adoptive transfer.

[0044] In addition to the method described above involving the ex vivo treatment or education of cells, the present invention also provides for a method where the ex vivo treatment or education is accompanied by the method of directly administering to the subject to be treated, by a variety of ways, an effective amount of the intermediary metabolite, antigen presenting cells, and antigens or epitopes, or any combination of the above. The disease may also be treated by only the direct administration of an effective amount of the intermediary metabolite, antigen presenting cells, and antigens or epitopes, or any combination of the above.

[0045] A therapeutic composition for the use in the treatment of the disease may comprise an effective amount of the intermediary metabolite, antigen presenting cells, and antigens or epitopes, or any combination of the above.

[0046] The treatment of a disease in any of the described methods results in a change in the number or function of regulatory, immune-regulatory or NKT cells. This change encompasses a reduction, inhibition, or decrease in the number or function of the cells. This inhibition may be caused by the competitive displacement of activating elements from the CD1d molecule. A change may also include a stimulation or increase in the number or function of the cells. This stimulation may be caused by increased binding of the activating elements from the CD1d molecule.

[0047] The treatment of a disease may also result in a change the cytokine responses. Any cytokine in the immune system may be involved in these responses. The change could result in a pro-inflammatory or an anti-inflammatory response. There may also be a pro-inflammatory, and an anti-inflammatory response since certain cytokines may increase and others may decrease, simultaneously.

[0048] Another result of the treatment of a disease is an alteration of the regulatory, immune-regulatory or NKT cell distribution in the subject. This change may also be accompanied by a change in the peripheral/intrahepatic T cell ratio. A further result may also include a change in intrahepatic CD8+ T cell trapping. There may be an increase or a decrease in the intrahepatic trapping. The result may also include a change in intrasplenic T cell trapping, where said change could be an increase or decrease.

[0049] Also provided in the present invention are two in vitro screening assays for an analogue or derivative of an intermediary metabolite which is administered to the subject to treat a disease. The first assay involves providing regulatory, immune-regulatory or NKT cells from the subject being

treated or another subject, antigen presenting cells, and an analogue or derivative of the intermediary metabolite in vitro. If a decrease in the regulatory, immune-regulatory or NKT cell proliferation is identified, then that specific analogue or derivative is a treatment for disease.

[0050] The second assay involves providing in a first test tube, regulatory, immune-regulatory or NKT cells and BSA; in a second test tube, regulatory, immune-regulatory or NKT cells and the analogue or derivative of an intermediary metabolite; in a third test tube, regulatory, immune-regulatory or NKT cells, antigen presenting cells and BSA; and in a fourth test tube, regulatory, immune-regulatory or NKT cells, antigen presenting cells and the analogue or derivative of the intermediary metabolite. If the least amount of regulatory, immune-regulatory or NKT cell proliferation is found in the fourth test tube, then that specific analogue or derivative is a treatment for the disease.

[0051] In a preferred embodiment of the present invention, there is minimal interference with digestion and absorption of an intermediary metabolite, an analogue or derivative of an intermediary metabolite, a lipid, conjugated biomolecule, polar lipid, glycolipid, lipoprotein, apolipoprotein, cytokines or hormones, monosaccharide ceramide, glucosylceramide, galactosylceramide, glucocereboside, glucocereboside analogue or derivative, sphingosine, sphingolipid, ceramide, T cell ligand, T cell receptor ligand, a T cell receptor ligand analogue or derivative, or a glycoprotein other than an antibody, in the mammalian subject. Specifically, the mammalian subject has been without food and/or water for a certain amount of hours prior to the administration of the aforesaid molecules, treatment of the mammalian subject or the manipulation of cells in the mammalian subject.

[0052] The methods for carrying out the invention, and the experimental results which support and further explain the results obtained are as follows:

EXAMPLES

I. Glucocerebroside Treatment of Concanavalin-A Hepatitis

Materials and Methods

Reagents

[0053] Concanavalin A was purchased from Worthington biochemical corporation, USA.

[0054] Glucocerebroside (Glucosylceramide or Glucosylcerebroside) was purchased from Avanti Polar Lipids, Inc.

Animals

[0055] Five groups of male Balb/C mice (n = 6/group) were studied.

Serum Transaminase Measurement

[0056] Serum ALT and AST plasma activity were measured by an automated commercial kit (Kodak SMA).

Hepatic Histology Examination

[0057] Histological sections of the livers from all mice were examined to determine the degree of liver damage. For each mouse a single liver segment was fixed in 10% buffered formaldehyde and embedded in paraffin for histologic analysis. Sections were stained with hematoxylin/eosin and histologic evaluation was performed.

Measurement of Cytokine Levels

[0058] Blood was drawn from mice in all groups and centrifuged at 14,000 rpm. Serum IFNγ, IL2, IL4, IL10 and IL-12 levels were measured by "sandwich" ELISA using Genzyme Diagnostics kits (Genzyme Diagnostics, MA).

Splenic and Hepatic Lymphocyte Isolation

[0059] Splenocytes were isolated and red blood cells removed as previously described [Vicari, A.P., *et al.*, Immunology Today 17(2):71 (1996)]. Intrahepatic lymphocytes were isolated from all groups of mice at the end of the study, as previously described, with some modifications [Vicari *et al.*, (1996) *ibid.*; Bleicher, P.A., *et al.*, Science 250:679-682 (1990)]. The inferior vena cava was cut above the diaphragm and the liver was flushed with 5 ml of cold PBS until it became pale. The connective tissue and the gall bladder were removed, and livers were place in a 10-ml dish in cold sterile PBS. Livers and spleens were crushed through a stainless mesh (size 60, Sigma Chemical Co., St. Louis MO). Cell suspension was placed in a 50 ml tube for 3 minutes and washed twice in cold PBS (1,250xrpm for 10 minutes), and debris was removed. Cells were re-suspended in PBS, cell suspension was placed through a nylon mesh presoaked in PBS, and unbound cells were collected. Cells were washed twice in 45 ml PBS (1,250xrpm in room temperature). For liver and spleen lymphocyte isolation 20 ml of histopague 1077 (Sigma Diagnostics, St. Louis, MO) were slowly placed underneath the cells suspended in 7 ml of PBS, in a 50-ml tube. The tube was centrifuged at 1,640 rpm for 15 minutes at room temperature. Cells at the interface were collected, diluted in a 50-ml tube, and washed twice with ice-cold PBS (1,250 rpm for 10 minutes). Approximately $1x10^6$ cells/mouse liver were recovered. The viability by trypan blue staining was more than 95%. Both splenocytes and liver-associated lymphocytes were isolated from all animals in all experimental groups.

Flow Cytometry Analysis for NKT Lymphocytes in Peripheral Blood

[0060] Immediately following intrahepatic and intrasplenic lymphocyte isolation, triplicates of $2\text{-}5x10^4$ cells/500µl PBS were put into Falcon 2052 tubes incubated with 4 ml of 1% BSA for 10 minutes, and centrifuged at

1400 rpm for 5 minutes. Cells were resuspended in 10µl FCS with anti-NK1.1 and anti-CD3 antibodies (Pharmingen, USA) and mixed every 10 minutes for 30 minutes. Cells were washed twice in 1% BSA, and kept in 4°C until reading. For the control group, only 5µl of 1% BSA was added. Analytical cell sorting was performed on $1x10^4$ cells from each group with a fluorescence-activated cell sorter (FACSTAR plus, Becton Dickinson). Only live cells were counted, and background fluorescence from non-antibody-treated lymphocytes were deducted from the levels obtained. Gates were set on forward- and side-scatters to exclude dead cells and red blood cells. The data were analyzed with Consort 30 two-color contour plot program (Becton Dickinson, Oxnard, CA), or the CELLQuest program.

Example 1

Glucocerebroside Amelioration of Concanavalin-A Hepatitis by the Inhibition of NKT Regulatory Lymphocytes

[0061] To evaluate the immune modulatory effect of Glucocerebroside on Concanavalin-A (Con-A) induced hepatitis, five groups of Balb/C mice, consisting of 6 mice each were studied. Group A and Group B were treated intraperitoneally with 1µg Glucocerebroside two hours prior to and two hours following, respectively, the intravenous administration of 500µg of Con-A. Group C mice received only 500µg of Con-A, and no Glucocerebroside. Group D mice were treated with 1µg Glucocerebroside, and no Con-A. Group E mice were naïve controls. This is summarized in Table 1.

Table 1

Experimental and Control Groups

Group	ConA (IV 500ug)	Glucocerebroside (IP 1ug)
A	+	+ (2 hours before ConA)
B	+	+ (2 hours after ConA)
C	+	-
D	-	+
E	-	-

[0062] Treatment with Glucocerebroside significantly ameliorated Con-A induced hepatitis, as shown in Figure 3 by markedly reduced serum AST and ALT levels. Group A had an ALT level of 57 IU. Group B and Group C had ALT levels of 420 IU and 801 IU, respectively. Group A had an AST level of 143 IU. Group B and Group C had AST levels of 559 IU and 600 IU, respectively. The administration of Glucocerebroside alone in Group D did not show a significant change in AST or ALT levels compared to Group E, the naïve control.

[0063] As shown in Table 2, treatment with Glucocerebroside two hours before Con-A administration in Group A resulted in normal results in almost all biopsies. Group B and Group C mice showed ischemia, necrosis and apoptosis. As shown in Figure 4, liver histological sections prepared from Group A and Group B mice revealed markedly attenuated damage compared to sections prepared from Group C livers, in which massive hepatocyte damage and characteristic apoptosis related changes were present.

Table 2

Effect of Glucocerebroside on Liver Pathology

A	Normal in almost all biopsies
B	Ischemia, necrosis, apoptosis
C	Ischemia, necrosis, apoptosis
D	Normal
E	Normal

[0064] Figure 5 shows that Glucocerebroside treatment significantly lowered serum IFNγ levels. Group A had approximately 3,725 pg/ml and Group C had 5,620 pg/ml. Figure 6 shows that serum IL2 levels increased with Glucocerbroside treatment: Group A had approximately 602 pg/ml and Group C had 206 pg/ml. Serum IL12 levels, as shown in Figure 7, also increased with Glucocerebroside: Group A had approximately 22,250 pg/ml and Group C had 10,100 pg/ml. As shown in Figures 8 and 9, respectively, serum IL4 and IL10 levels decreased with Glucocerebroside treatment. According to Figure 8, Group A had a serum IL4 level of approximately 31 pg/ml and Group C had 37 pg/ml. According to Figure 9, Group A had a serum IL10 level of approximately 8 pg/ml and Group C had 26 pg/ml.

[0065] As shown in Figure 10, the effect of Glucocerebroside on immune mediated hepatitis was associated with a significant decrease in intrahepatic NKT lymphocytes. Such a decrease did not occur with intrasplenic NKT lymphocytes (see Figure 11).

[0066] In Figure 12, the proliferation of NKT cells containing various components in vitro were studied. Group A contained NKT cells and BSA; Group B contained NKT cells and Glucocerebroside; Group C contained NKT cells, Dentritic Cells and BSA; and Group D contained NKT cells, Dendritic

Cells and Glucocerebroside. The stimulation index decreased from Group A to Group D. This depicts that there is an overall decrease in NKT cell proliferation. The presence of Glucocerebroside and Dentritic Cells is necessary for this NKT cell decrease.

[0067] The administration of Glucocerebroside resulted in the significant amelioration of Con-A hepatitis. This effect was accompanied by a significant decrease in the IFNy response. These results suggest that the Glucocerebroside effect may be associated with the inhibition of intrahepatic NKT cells by the competitive displacement of activating elements from the CD1d molecule.

II. Glucocerebroside Treatment of Colitis

Materials and Methods

Animals

[0068] Normal inbred 2 to 4 month old Balb/c male mice were obtained from Jackson Laboratories, USA and maintained in the Animal Core of the Hadassah-Hebrew University Medical School. Mice were maintained on standard laboratory chow and kept in 12-hour light/dark cycles.

Induction of Colitis

[0069] 2,4,6-trinitrobenzene sulfonic acid (TNBS) - colitis was induced by rectal instillation of TNBS, 1 mg/mouse, dissolved in 100 ml of 50% ethanol as described. [Collins, C., *et al.*, Eur. J. Immunol. 26:3114-3118 (1996)].

Evaluation of the Effect of Glucocerebroside on Experimental Colitis

[0070] The effect of Glucocerebroside was evaluated by monitoring the following parameters for colitis:

Clinical Assessment of Colitis:

[0071] Diarrhea was followed daily throughout the study.

Macroscopic Score of Colitis

[0072] Colitis assessment was performed 14 days following colitis induction using standard parameters [Madsen, K.L., *et al.*, Gastroenterology 113:151-159 (1997); Trop, S., *et al.*, Hepatology 27:746-755 (1999)].

[0073] Four macroscopic parameters were determined, namely: diarrhea, degree of colonic ulcerations; intestinal and peritoneal adhesions; and wall thickness. Each parameter was graded on a scale from 0

(completely normal) to 3 (most severe) by two experienced blinded examiners.

Grading of Histological Lesions

[0074] For histological evaluation of inflammation, distal colonic tissue (last 10 cm) was removed and fixed in 10% formaldehyde. Five paraffin sections from each mouse were then stained with hematoxylin-eosin by using standard techniques. The degree of inflammation on microscopic cross sections of the colon was graded semiquantitatively from 0 to 4 [Madsen *et al.*, (1997) *ibid.*; Trop *et al.*, Hepatology 27:746-755 (1999)]. Grade 0: normal with no signs of inflammation; Grade 1: very low level of leukocyte infiltration; Grade 2: low level of leukocyte infiltration; and Grade 3: high level of infiltration with high vascular density, and bowel wall thickening; Grade 4: transmural infiltrates with loss of goblet cells, high vascular density, wall thickening, and disruption of normal bowel architecture. The grading was performed by two experienced blinded examiners.

Splenic and Hepatic Lymphocyte Isolation

[0075] Splenocytes were isolated and red blood cells removed as previously described [Vicari, A.P., *et al.*, Immunology Today 17(2):71 (1996)]. Intrahepatic lymphocytes were isolated from all groups of mice at the end of the study, as previously described, with some modifications [Vicari *et al.*, (1996) *ibid.*; Bleicher, P.A., *et al.*, Science 250:679-682 (1990)]. The inferior vena cava was cut above the diaphragm and the liver was flushed with 5 ml of cold PBS until it became pale. The connective tissue and the gall bladder were removed, and livers were place in a 10-ml dish in cold sterile PBS. Livers and spleens were crushed through a stainless mesh (size 60, Sigma Chemical Co., St. Louis MO). Cell suspension was placed in a 50 ml tube for 3 minutes and washed twice in cold PBS (1,250xrpm for 10 minutes), and debris was removed. Cells were re-

suspended in PBS, cell suspension was placed through a nylon mesh presoaked in PBS, and unbound cells were collected. Cells were washed twice in 45 ml PBS (1,250xrpm in room temperature). For liver and spleen lymphocyte isolation 20 ml of histopague 1077 (Sigma Diagnostics, St. Louis, MO) were slowly placed underneath the cells suspended in 7 ml of PBS, in a 50-ml tube. The tube was centrifuged at 1,640 rpm for 15 minutes at room temperature. Cells at the interface were collected, diluted in a 50-ml tube, and washed twice with ice-cold PBS (1,250 rpm for 10 minutes). Approximately $1x10^6$ cells/mouse liver were recovered. The viability by trypan blue staining was more than 95%. Both splenocytes and liver-associated lymphocytes were isolated from all animals in all experimental groups.

FACS of Intrahepatic and Intrasplenic Lymphocytes for NKT, CD4 and CD8 Markers

[0076] Immediately following lymphocyte isolation, triplicates of 2-$5x10^4$ cells/500µl PBS were put into Falcon 2052 tubes incubated with 4 ml of 1% BSA for 10 minutes, and centrifuged at 1400 rpm for 5 minutes. Analysis of lymphocyte subpopulations was performed using anti-NK1.1, anti-CD3, anti-CD4 and anti CD-8 antibodies. Cells were washed twice in 1% BSA, and kept in 4°C until reading. For the control group, only 5µl of 1% BSA was added. Analytical cell sorting was performed on $1x10^4$ cells from each group with a fluorescence-activated cell sorter (FACSTAR plus, Becton Dickinson). Only live cells were counted, and background fluorescence from non-antibody-treated lymphocytes were deducted from the levels obtained. Gates were set on forward- and side-scatters to exclude dead cells and red blood cells. The data were analyzed with Consort 30 two-color contour plot program (Becton Dickinson, Oxnard, CA), or the CELLQuest program.

Measurement of Cytokine Levels

[0077] Blood was drawn from mice in all groups and centrifuged at 14,000 rpm. Serum IFNγ, IL2, IL4, IL10 and IL-12 levels were measured by "sandwich" ELISA using Genzyme Diagnostics kits (Genzyme Diagnostics, MA).

Example 1

Glucocerebroside Amelioration of Experimental Colitis

[0078] To evaluate the immune modulatory effect of Glucocerebroside in a murine model of experimental colitis, four groups of Balb/c mice, consisting of 10 mice each were studied. Group A and Group B mice were challenged with rectal TNBS and Group C and Group D were given normal saline. Group B and Group D mice were intraperitoneally administered 1.5μg of Glucocerebroside daily, for 9 days. This is summarized in Table 3.

Table 3

Experimental and Control Groups

Group	TNBS	Glucocerebroside (IP 1.5ug)
A	+	-
B	+	+
C	-	-
D	-	+

[0079] As shown in Figure 14, treatment with Glucocerebroside showed improvement in the macroscopic colitis score for Diarrhea. Group A had a score of approximately 0.22 and Group B had a score of approximately 0.5. The score for the degree of colonic ulcerations also improved, since Group A had an approximate score of 0.11 and Group B had an approximate score of 0.2. There was also a slight improvement in macroscopic score for wall thickness, since both Group A and Group B had approximate scores of 2.44 and 2.56, respectively. However, intestinal and peritoneal adhesions increased for Group A, versus Group B, where approximate scores were 2.56 and 1.4, respectively.

[0080] As shown in Figure 13 and Figure 15, Group A, which did not receive Glucocerebroside, had the highest microscopic colitis score of approximately 3.6, evidencing a high degree of inflammation. Group B, C and D had practically normal biopsies (lower microscopic scores).

[0081] The administration of Glucocerebroside resulted in marked alleviation of colitis, manifested by significant improvement of the macroscopic and microscopic colitis scores in Group A mice compared to Group B mice.

[0082] The effect of Glucocerebroside on Group C and Group D mice showed a Spleen CD4/CD8 ratio of 3.0 and 1.89, respectively. The effect of Glucocerebroside on Group C and D mice showed a liver CD4/CD8 ratio of 8.8 and 3.4, respectively. The ratio of ratios of Group C mice (naïve animals) versus Group D mice (animals treated with Glucocerebroside) were 0.34 and 0.65, respectively. These results show a decrease in NKT cells in the periphery and the liver, and a decreased CD4/CD8 ratio in the periphery and the liver. Therefore, the effect of Glucocerebroside was more intrahepatic CD8 trapping. These results are shown in Figure 16 and Figure 17.

[0083] The effect of Glucocerebroside on Group A and Group B mice showed a Spleen CD4/CD8 ratio of 1.89 and 3.33, respectively. The effect of Glucocerebroside on Group A and Group B mice showed a liver CD4/CD8 ratio of 5.0 and 5.24, respectively. The ratio of ratios of Group A mice (animals with colitis not treated with Glucocerebroside) versus Group B mice (animals with colitis treated with Glucocerebroside) were 0.34 and 0.65, respectively. These results show an increase in NKT cells in the periphery and no change in NKT cells in the liver. There was an increased peripheral CD4/CD8 ratio and a mild increase of the liver CD4/CD8 ratio. These results are also shown in Figure 16 and Figure 17. Glucocerebroside treatment resulted in more intrahepatic CD8 trapping.

[0084] Figure 18 shows the effect of Glucocerebroside on serum cytokine levels. Serum IFNγ levels were increased with Glucocerebroside treatment. Group A had approximately 8.3 pg/ml and Group B had approximately 27.1 pg/ml. Serum TNFa levels also increased with Glucocerbroside treatment: Group A had approximately 75 pg/ml and Group B had approximately 103.6 pg/ml. Serum IL4 levels also increased with Glucocerebroside: Group A had approximately 5.7 pg/ml and Group B had approximately 9.1 pg/ml. However, serum IL10 levels decreased with Glucocerebroside treatment. Group A had a serum IL10 level of approximately 42.1 pg/ml and Group B had approximately 21.4 pg/ml.

[0085] Alleviation of colitis by Glucocerebroside treatment was associated with a significant increase in intrahepatic $CD8^+$ T cell trapping. The peripheral/intrahepatic $CD4^+/CD8^+$ ratio increased by 85% in Group A mice treated with Glucocerebroside versus untreated Group B mice. A similar effect was observed when Glucocerebroside was administered to naïve animals: the peripheral/intrahepatic $CD4^+/CD8^+$ ratio increased by

61% in Group C Glucocerebroside treated mice versus untreated animals. While Glucocerebroside treatment led to a 108% increase of the peripheral/intrahepatic NKT cell ratio in naïve mice, the beneficial effect of Glucocerebroside on TNBS colitis was associated with a relative decrease of this ratio.

[0086] Similar results were obtained when the same experiment was conducted with the15μg of Glucocerebroside, administered orally. There was a marked alleviation of colitis manifested by a significant improvement of the macroscopic and microscopic colitis scores in Group A Glucocerebroside treated mice compared to the untreated Group B mice, as shown in Table 4.

Table 4

Microscopic and Macroscopic Results of the Oral Administration of Glucocerebroside for the Treatment of Colitis

Microscopic

Mouse No.	A (TNBS)	B (TNBS+15μgGC)	C (Naïve)	D (Naïve + 150μgGC)
1	3	0.5	0.5	1
2	3.5	1.5	1.5	1
3		0.5		0.5
4	2		0.5	2
5	4	1.5	1.5	0
6	2	1.5	0.5	0
7	2.5	0	0.5	0.5
8	4		1	0
9		2	0.5	0
10			2	1

Macroscopic

Mouse No.	A (TNBS)	B (TNBS+15μgGC)	C (Naïve)	D (Naïve + 150μgGC)
1	0	0.5	0	0.5
2	0.5	0	0	0.5
3	1	0.5	0	0
4	1.5	0.5	0	0
5	1.5	0.5	0	0
6	3.5	0.5	0	0
7	2.5	0.5	0	0
8		0	0	0
9		0	0	0
10		0	0	0

III. Glucocerebroside Treatment of Non-Alcoholic Steatohepatitis

Materials and Methods

Animals

[0087] Ten-week-old male leptin-deficient C57BL/6J mice and lean C57BL/6 mice were purchased from Harlan laboratories and maintained in the Animal Core of the Hadassah-Hebrew University Medical School. Mice were fed standard laboratory chow and kept in 12-hour light/dark cycles.

Glucose Tolerance Test

[0088] Glucose tolerance was assessed by oral administration of glucose (1 gram per kilogram body weight). Blood drawn from the tail was measured for glucose at 0', 15', 30', 60', 90', 120' and 180'. Glucose levels were measured with Elite glucose test strips and a glucometer.

Hepatic MRI Measurement of Fat Content

[0089] Hepatic fat content was measured using a double-echo chemical shift gradient-echo magnetic resonance imaging (MRI) sequence that provides in-phase and opposed-phase images in a single acquisition for assessment/quantification of fat in mouse liver. The T1-weighted opposed-phase MR imaging technique is sensitive for detection of relatively small amounts of tissue fat. MRI images were performed with a 1.5-T system (Signa LX;GE, Milwaukee, USA). Double-echo MR imaging was performed with a repetition time (TR) of 125 msec, double echo times (TEs) of 4 and 6.5 msec, and a flip angle of 80°. Imaging parameters included section thickness of 3mm, 13-cm field of view, 256*160 matrix, and one signal acquired, with use of a knee coil. Transverse (axial) and coronal images were acquired at the level of the liver with a 3mm section thickness and no intersection gap. Quantitative assessment of signal intensity (SI) measurements of SI changes between in-phase and opposed-phase images was computed as described in previous reports (Mitchell DG et al., Invest.

Radiol 26:1041-1052 (1991); Tomohiro N et al., Radiology 218:642-646 (2001)). The SI index was calculated as follows: SI index = $(SI_{ip}-Si_{op})/SI_{ip}$, where SI_{ip} is SI on in-phase images and SI_{op} is SI on opposed-phase images. The SI index reflects the fraction of SI loss on opposed phase images compared with the SI on in-phase images.

Example 1

Effect of Glucocerebroside on Diabetes

[0090] To evaluate the effect of Glucocerebroside on the various metabolic and immunologic components of the NASH model, four groups of C57bl mice, consisting of 12 mice each were studied. As shown in Table 5, Group A and Group B mice were ob/ob mice, whereas Group C and Group D mice were not. Group A and Group C mice were injected intraperitoneally with 1.5 μg in 100 μl PBS every other day for 14 days. Group B and Group D naïve ob/ob mice and naïve C57bl mice, respectively, were left untreated.

Table 5

Experimental and Control Groups

A	**OB / OB MICE** INJECT WITH GLUCOCEREBROSIDE **IP 1.5μg**/mouse in 100 μl PBS every other day
B	Naive **OB / OB MICE** untreated
C	C57bl INJECT WITH GLUCOCEREBROSIDE **IP 1.5μg**/mouse in 100 □l PBS every other day
D	Naive C57bl untreated

[0091] On the 14th day, glucose tolerance tests were performed on 6 mice from each group. As depicted in Figure 19, Group A mice, which were treated with Glucocerebroside, had a higher glucose tolerance than naïve ob/ob mice that were not treated. This suggests that Glucocerebroside

injection alters the metabolic profile of ob/ob mice, improving their glucose tolerance results, rendering them less diabetic.

Example 2

Effect of Orally Administered Glucocerebroside on NASH

[0092] To evaluate the effect of Glucocerebroside on the various metabolic and immunologic components of the NASH model, four groups of C57bl mice, consisting of 12 mice each were studied. As shown in Table 6, Group A and Group B mice were ob/ob mice, whereas Group C and Group D mice were not. Group A and Group C mice were injected intraperitoneally with 1.5 μg in 100 μl PBS every other day for 14 days. Group B and Group D naïve ob/ob mice and naïve C57bl mice, respectively, were left untreated.

Table 6

Experimental and Control Groups

A	**OB / OB MICE** FEED GLUCOCEREBROSIDE 15μg/mouse in 100 μl PBS every other day
B	Naive **OB / OB MICE** untreated
C	C57bl FEED GLUCOCEREBROSIDE 15μg/mouse in 100 μl PBS every other day
D	Naive C57bl untreated

[0093] On the 14th day, glucose tolerance tests were performed on 6 mice from each group. As depicted in Figure 20, Group A mice, which were treated with Glucocerebroside, had a higher glucose tolerance than naïve ob/ob mice that were not treated. This suggests that immune modulation

through oral immune regulation induction alters the metabolic profile of ob/ob mice, improving their glucose tolerance results, rendering them less diabetic.

Example 3

The Effect of Glucocerebroside on the Hepatic Fat Content

[0094] To determine the effect of Glucocerebroside on the various metabolic and immunologic components of the NASH model, four groups of C57bl mice, consisting of 12 mice each were studied. As shown in Table 7, Group A and Group B mice were ob/ob mice, whereas Group C and Group D mice were not. Group A and Group C mice were injected intraperitoneally with 1.5 μg in 100 μl PBS every other day for 14 days. Group B and Group D naïve ob/ob mice and naïve C57bl mice, respectively, were left untreated.

Table 7

Experimental and Control Groups

A	**OB / OB MICE** INJECT WITH GLUCOCEREBROSIDE **IP 1.5μg**/mouse in 100 μl PBS every other day
B	Naive **OB / OB MICE** untreated
C	C57bl INJECT WITH GLUCOCEREBROSIDE **IP 1.5μg**/mouse in 100 μl PBS every other day
D	Naive C57bl untreated

[0095] To determine hepatic fat content, mice of all four groups underwent an abdominal MRI on day 14 of the experiment (Table 8). Hepatic fat content was determined and was described as the SI index (IP-OP/IP). Liver size, in area, was also determined. The results showed a reduction in liver fat content due to Glucocerebroside treatment. Group A mice treated

with Glucocerebroside had an SI index of 0.46, as compared to Group B, which had an SI index of 0.54. There was also a reduction in liver size resulting form Glucocerebroside treatment. Glucocerebroside treated Group A mice had a liver area of 20.14, as compared to Group B, which had a liver area of 24.2.

Table 8

Calculated MRI Hepatic Fat Content of the Six Mice Groups

	In Phase Images	Opposite Phase Images	FAT CONTENT (IP-OP)	SI INDEX (IP-OP/IP)	Area
Fat	536	351	185	0.35	25
	603	293	310	0.51	16
	575	251	324	0.56	20.5
	554	234	320	0.58	23.5
	520	202	378	0.61	30.5
	560	201	359	0.64	28.5
Average				0.54	24.2

	In Phase Images	Opposite Phase Images	FAT CONTENT (IP-OP)	SI INDEX (IP-OP/IP)	Area
Fat + Tx	514	279	235	0.46	13.5
	527	256	271	0.51	20
	574	305	269	0.47	26
	561	344	217	0.39	18.5
	462	283	179	0.39	21.5
	579	309	270	0.47	27.5
	1132	502	629	0.56	14
Average			- 30 -	0.464286	20.14286

	In Phase Images	Opposite Phase Images	FAT CONTENT (IP-OP)	SI INDEX (IP-OP/IP)	Area
Thin	518	423	95	0.18	10.5
	517	434	83	0.16	11.5
	476	397	79	0.17	11.5
	1040	813	227	0.22	10
	892	731	161	0.18	10
Average				0.18	10.7

	In Phase Images	Opposite Phase Images	FAT CONTENT (IP-OP)	SI INDEX (IP-OP/IP)	Area
Thin + Tx	547	479	68	0.12	16.5
	443	424	19	0.04	14
	472	409	63	0.13	8
	507	440	67	0.13	16.5
	532	438	94	0.18	5.5
	534	481	53	0.1	10
	987	871	117	0.12	15
	974	839	135	0.14	13.5
	930	870	60	0.06	9
	302	787	115	0.13	8
	927	889	40	0.04	15
	910	887	23	0.03	10
Average				0.1	11.75

[0096] This suggests that Glucocerebroside alters the metabolic profile in a way which results in a reduction in the rate of fat accumulation and NASH in the livers of susceptible mammals.

IV. Glucocerebroside Treatment of Melanoma

Materials and Methods

Animals

[0097] Four groups of C57bl mice were studied.

Histology Examination

[0098] Histological sections of the lungs from mice were examined to determine the degree of lung damage. For each mouse a single lung segment was fixed in 10% buffered formaldehyde and embedded in paraffin for histologic analysis. Sections were stained with hematoxylin/eosin and histologic evaluation was performed.

Example 1

Effect of Glucocerebroside Treatment on Melanoma

[0099] To evaluate the effect of Glucocerebroside on melanoma, four groups of C57bl mice, consisting of 8 mice each were studied. Group A and Group B were subcutaneously administered 1×10^6 cells of the B16 melanoma cell line and Group C and Group D were intravenously treated with 1×10^5 cells of the B16 melanoma cell line to induce melanoma. Group A and Group C were treated with 1μg of Glucocerebroside intraperitoneally, every day, skipping the last two days of every week, starting on the second day of the first week. Group B and Group D mice were given saline only. This is summarized in Table 9.

Table 9

Experimental and Control Group

Group:		Melanoma
A	GC treatment	SC
B	SALINE	SC
C	GC treatment	IV
D	SALINE	IV

[0100] Treatment with Glucocerebroside significantly ameliorated tumor size. Tumors were removed and subsequently measured. The average tumor weight in Group A was 1.63 +/- 0.82g, and the average tumor weight in Group B was 2.89 +/- 0.01g. The differences in tumor size can be seen in Figure 21.

[0101] Treatment with Glucocerebroside also showed a decrease in lung metastasis. Lung cells of Group C and Group D were fixed for histological analysis. The average number of lung metastasis in Group C was 3 +/- 1 per lung and the mean number of lung metastasis in Group D was 8 +/- 3 per lung.